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Inheritance of Functional Foreign Genes in Plants

Abstract. *Morphologically normal plants were regenerated from* Nicotiana plumbaginifolia *cells transformed with an* Agrobacterium tumefaciens *strain containing a tumor-inducing plasmid with a chimeric gene for kanamycin resistance. The presence of the chimeric gene in regenerated plants was demonstrated by Southern hybridization analysis, and its expression in plant tissues was confirmed by the ability of leaf segments to form callus on media containing kanamycin at concentrations that were normally inhibitory. Progeny derived from several transformed plants inherited the foreign gene in a Mendelian manner.*

Agrobacterium tumefaciens, the causative agent of crown gall disease, is capable of transferring a DNA segment (designated T-DNA), located between specific border sequences, from its tumor-inducing plasmid (Ti plasmid) into the nuclear DNA of infected plant cells (*1*). Expression of T-DNA-encoded tumor genes in the transformed cell provides a selectable trait for recognition of those cells in culture; namely, the ability to grow on medium without added phytohormones. Unfortunately, this trait interferes with regeneration of normal fertile transformed plants (*2*).

Recently, we (*3*) and others (*4*) have constructed chimeric genes that function as dominant selectable markers in plant cells, thus making the tumor genes unnecessary for identification of transformants. Our chimeric gene contains the coding sequence of the bacterial gene for neomycin phosphotransferase II (NPTII) joined to the 5′ and 3′ regulatory regions of the nopaline synthase (NOS) gene, which is expressed constitutively in higher plant cells (*5*). We have shown that petunia and tobacco cells transformed with this chimeric NOS/NPTII/NOS gene are readily selected and are highly resistant to kanamycin (*3*).

We now report that kanamycin-resistant plant cells obtained with our vector system regenerate to morphologically normal plants. These plants carry a functional kanamycin-resistance gene and produce viable seeds. Analysis of progeny shows that the chimeric kanamycin-resistance gene is inherited and is expressed as a dominant Mendelian trait.

We used the previously described pMON120 intermediate vector to introduce the chimeric NOS/NPTII/NOS gene into the *A. tumefaciens* Ti plasmid. The pMON120 plasmid also contains an intact NOS gene as a scorable transformation marker. This NOS fragment includes the nopaline-type T-DNA right border sequence (*6*). Because this additional border sequence is initially carried on a separate plasmid, we refer to our system as the split end vector (SEV) system. Figure 1 shows how this system is used. The pMON128 plasmid [pMON120 containing the chimeric kanamycin-resistance gene (Fig. 1B)] is introduced by conjugation into *A. tumefaciens* cells, where homologous recombination with a resident octopine-type Ti plasmid [pTiB6S3 (Fig. 1A)] occurs. The resultant cointegrate plasmid pTiB6S3::pMON128 (Fig. 1C) contains a hybrid T-DNA in which the nopaline-type right border sequence is positioned between the kanamycin-resistance gene and the tumor genes of the resident Ti plasmid. Use of the nopaline T-DNA border sequence during infection results in the transfer of a short T-DNA segment (Fig. 1E) which contains the kanamycin-resistance gene and an intact NOS gene but does not contain genes for tumor formation or octopine synthase (*7*). The short-transfer transformants can be regenerated to give intact plants as described below.

Transformation of *Nicotiana plumbaginifolia* cells was carried out with the engineered *A. tumefaciens* train containing the chimeric NOS/NPTII/NOS gene by the method of cocultivation (*3*).

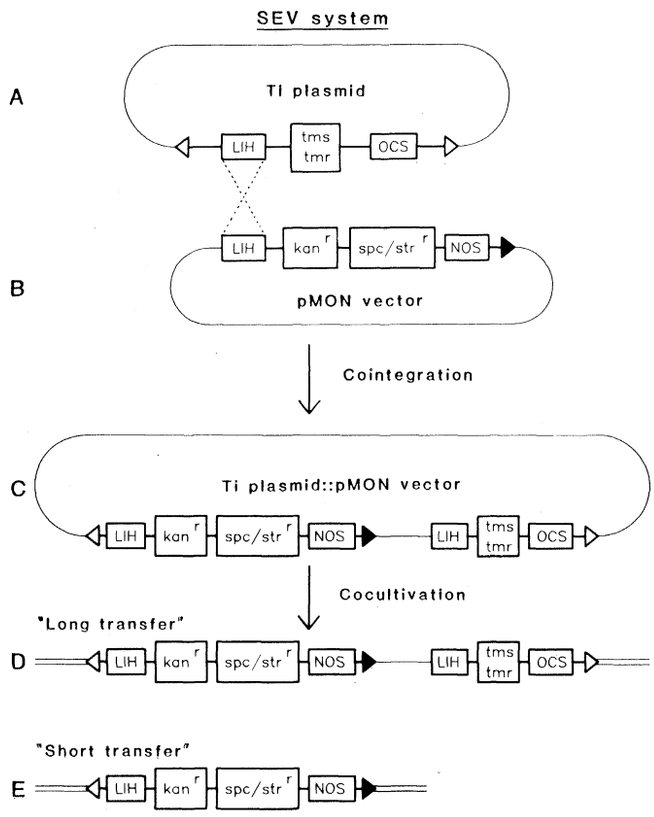

Fig. 1. Steps in the SEV system for plant cell transformation. The arrows represent the T-DNA border sequences. *LIH* is a region of homologous DNA for recombination. The tumor genes are represented by *tms* and *tmr* (*11*); *OCS* and *NOS* are octopine and nopaline synthase genes, respectively. The chimeric kanamycin-resistance gene is designated as *kan*^{r}. The bacterial spectinomcyin - streptomycin resistance determinant for selection of cointegrates is designated *spc/str*^{r}. Reciprocal recombination of (A) a resident Ti plasmid (pTiB6S3) and (B) pMON120 derivative (pMON128) yields (C) the cointegrate, pTiB6S3::pMON128. After cocultivation and selection for kanamycin-resistant plant cells either (D) the entire hybrid T-DNA or (E) a truncated T-DNA without tumor genes is transferred into the plant genome.

Leaf mesophyll protoplasts of *N. plumbaginifolia* were obtained and cultured as described by Pollock *et al.* (*8*). Two-day-old cultures of protoplast-derived cells were inoculated with live *A. tumefaciens* cells (10^7 cells per milliliter). After 48 hours of coculture, the plant cells were collected by centrifugation (100*g* for 5 minutes), washed twice, and replated in medium containing carbenicillin (500 μg/ml) to kill the remaining bacteria. On the sixth day after isolation of protoplasts, the procedure described for petunia cells (*3*) was used to transfer the cultures from liquid to feeder plates (*9*) of the same medium. After 7 to 10 days on the feeder plates, the colonies were transferred to fresh medium (without feeder cells). After another 7 days, colonies were transferred to a different medium [MS salts (Gibco), B5 vitamins, 3 percent sucrose, carbenicillin (500 μg/ml), kanamycin (100 μg/ml), benzyl adenine (1.0 μg/ml), and naphthalene acetic acid (0.1 μg/ml); *p*H 5.7]. After 7 days, the most promising colonies (largest and greenest) were picked from the filter paper substrate and transferred to the same medium at low density [10 colonies per petri plate (100 by 15 mm)].

In our first experiment, four kanamycin-resistant colonies were recovered from approximately 8×10^4 protoplasts cocultivated with pTiB6S3::pMON128. No resistant colonies were found among the same number of untreated control colonies. Southern blot hybridization with an NPTII specific probe showed that all four resistant colonies contained the chimeric gene (data not presented). One of the four colonies was morphogenic and produced a kanamycin-resistant plant, NPK3.

Leaf segments from NPK3 were able to form callus and proliferate new shoots on medium containing kanamycin (100 μg/ml) (Fig. 2A). In contrast, leaf segments from wild-type plants were completely inhibited. Analysis with Southern blot hybridization showed that the 1.5-kilobase (kb) Eco RI fragment containing the chimeric NOS/NPTII/NOS gene was present in the leaves of NPK3 but not in tissue from wild-type plants (lanes c and d in Fig. 2B).

Twenty-one first-generation progeny plants (S_1) from the self-fertilized transformed parent NPK3 were grown to maturity and tested for kanamycin resistance in the leaf callus assay. Fifteen of the 21 were able to form callus on medium with kanamycin (100 μg/ml). Another 80 seedlings (germinated under sterile conditions) were transferred to medium containing kanamycin (100 μg/ml). Of these, 62 grew several times larger and formed callus, whereas 18 ceased growth and did not form callus. Thus the trait was inherited in a Mendelian manner with a 3:1 ratio. The final proof of the correspondence between the presence of the chimeric gene and the antibiotic-resistant phenotype was established by the perfect correlation between inheritance of the chimeric gene and the kanamycin-resistant phenotype in S_1 plants (lanes e to n in Fig. 2B).

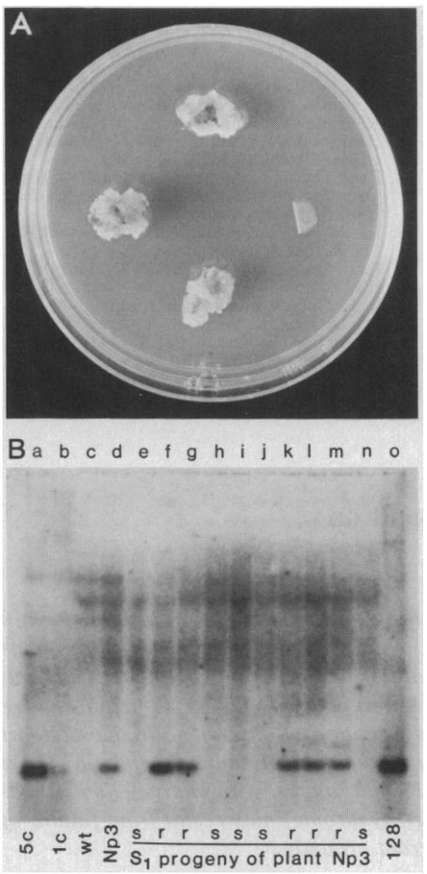

Fig. 2. Analysis of transformed progeny. (A) Leaf callus assay. Surface-sterilized segments of leaves were placed on medium [MS salts (Gibco), B5 vitamins, 3 percent (weight to volume) sucrose, benzyl adenine (1 μg/ml), and naphthalene acetic acid (0.1 μg/ml), *p*H 5.7] containing kanamycin (100 μg/ml). Explants from wild-type plants were unable to grow on this medium, whereas explants from our transformed plants callused and generated shoots within 3 weeks (data not shown). The explants shown here are from four separate S_1 progeny of NPK3. One of the progeny plants is clearly sensitive to kanamycin, whereas the other three are resistant. DNA blot hybridization analysis. (B) Total plant DNA was extracted, purified by CsCl gradient centrifugation, and digested (10 μg) with the restriction enzyme Eco RI as described (*3*). After transfer of the DNA to nitrocellulose, a nick-translated DNA probe specific for transposon Tn*5* was used to identify a fragment containing the chimeric NOS/NPTII/NOS gene (*3*). (Lanes a and b) Five-copy (*5c*) and one-copy (*1c*) reconstruction experiments; (lane c) DNA from wild-type (*wt*) control plants; (lane d) DNA from parental NPK3 plant (*Np3*); (lanes e to n) DNA from S_1 progeny of NPK3 plant; and (lane o) digested pMON128 plasmid showing the position of the 1.5-kb fragment of the chimeric gene. The letters *r* and *s* denote kanamycin resistance and sensitivity, respectively, in the leaf callus assay.

Three subsequent cocultivation experiments gave high frequencies of transformation, averaging 6 percent of the total colonies or 1.2 percent of the total initial protoplasts plated. The control populations consistently failed to yield any kanamycin-resistant colonies. Most (about 90 percent) of the kanamycin-resistant colonies produced both octopine and nopaline and were nonmorphogenic, as expected for transformants arising when the octopine T-DNA right border was utilized (Fig. 1D). About 10 percent of the colonies were morphogenic, producing shoots that could be excised, rooted, and grown in soil. Of 22 plants examined, 9 were escapes or revertants that showed none of the markers of transformation. The other 13 plants produced nopaline, but not octopine, and were resistant to kanamycin as measured by a leaf callus–induction assay.

The S_1 progeny from three of the independently isolated, nopaline-producing and kanamycin-resistant plants (NPK7, NPK9, NPK10) were scored for nopaline content. In each case, the progeny showed normal Mendelian inheritance and expression of the inserted DNA segment: 71 of 105 progeny of NPK7, 39 of 48 progeny of NPK9, and 34 of 44 progeny of NPK10 produced nopaline. In addition, axenically grown seedlings from each of the transformants showed similar segregation for ability to form callus on medium containing kanamycin (100 μg/ml). For example, 37 of 51 progeny of NPK10 formed callus in the presence of kanamycin, and all 37 resistant progeny produced nopaline.

We have shown that (i) the chimeric NOS/NPTII/NOS gene is expressed in *N. plumbaginifolia*, (ii) the regenerated transformed plants are phenotypically normal and fertile, and (iii) normal Mendelian inheritance of an engineered gene can occur in the progeny of transformed plants. The genetic transmission of chimeric antibiotic-resistance genes has now been confirmed for the S_2 progeny of the NPK3 plant.

Normal Mendelian inheritance of the chimeric gene has also been demonstrated for petunia plants transformed with pMON120-type vectors (*10*). The availability of dominant selectable markers and transformation vectors that permit

the regeneration of phenotypically normal plants will greatly facilitate studies of gene expression and regulation in plants.

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Dependence of Thymus Development on Derivatives of the Neural Crest

Abstract. *Elimination of limited areas of the cephalic neural crest in stage 9 or 10 chick embryos markedly reduced the size of the thymus gland or resulted in its absence. Small thymic lobes contained both thymocytes and epithelial cells but showed delayed development. Parathyroid and thyroid glands sometimes were reduced in size or missing from the normal location on one or both sides. Heart defects were consistently present. Thymus development may depend on direct interaction of mesenchymal derivatives of the neural crest with pharyngeal epithelium. Multiple defects, such as the Di George syndrome, may result from failure of neural crest derivatives to migrate and interact properly.*

Aplasia or dysplasia of the thymus gland may be associated with abnormal development of other pharyngeal pouch derivatives, facial features, and the heart (*1*). The neural crest contributes connective tissue to the cephalic region and thymus (*2*). It was recently demonstrated that the cephalic neural crest contributes to development of the cardiac outflow tract; extirpation of the crest led to abnormalities of the heart and great vessels (*3*). The occurrence of clusters of congenital anomalies of head structures, heart, and thymus, which have in common the incorporation of contributions from the cephalic neural crest, led to the hypothesis that failure of sufficient quantities of cephalic neural crest cells to migrate and interact with these developing organs results in their abnormal development. In support of this hypothesis for the thymus, we report here that defective development of the thymus accompanies cardiac anomalies after extirpation of the cephalic neural crest in chick embryos.

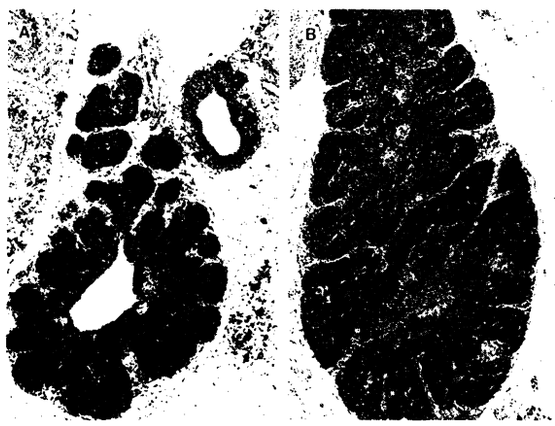

Fig. 1. Photomicrographs of sections of embryonic chick thymus taken (A) after extirpation of the cephalic neural crest and (B) after sham surgery (both ×100). Remnants of primitive pharyngeal pouch endoderm are evident in two areas in (A).

The neural crest is produced from ectoderm in the avian embryo after the neural folds appose each other to form the primitive neural tube. Neural crest cells migrate ventrolaterally from the area of the neural folds and differentiate to form a variety of structures, including cranial, spinal, and autonomic ganglia. The cephalic neural crest, that portion from fifth somite forward, also contributes a large proportion of cells that differentiate into mesenchymal cells. Bones and cartilage of the visceral skeleton, dermis in the face and ventrolateral side of the neck, walls of large arteries derived from branchial arches, and connective tissue of the lower jaw and tongue all originate from the neural crest (*2*, *4*). In addition, mesenchymal components of the glandular pharyngeal derivatives have the same origin. Cells derived from the neural crest form the parafollicular cells of the thyroid gland and are found in the interlobular spaces and medulla of thymic lobes and in the connective tissue between cords of parathyroid cells (*2*). The cephalic neural crest contributes to the formation of the periocular tissues and, as mentioned above, the outflow tract of the heart (*2*, *3*, *5*).

To determine whether thymus development depends on neural crest derivatives, we ablated regions of the neural folds of chick embryos before cephalic neural crest migration and subsequently examined the thymus. Fertile Arbor Acre chicken eggs (Central Soya of Athens) were incubated for 30 hours in a humidified atmosphere at 38°C. A window was made in the shell and the embryos, at stage 9 or 10 (*6*), were lightly stained with neutral red. The vitelline membrane was torn over the embryos and all or part of the neural folds over somites 1 to 5 were removed bilaterally with a modified Wenger vibrating needle (*7*) or a microcautery needle. Histological monitoring has shown that this technique causes minimal damage, limited to a few cell widths in the dorsal neural tube and adjacent surface ectoderm. After surgery the eggs were sealed, returned to the incubator, and allowed to develop for an additional 13 to 15 days (total incubation age, 14 to 16 days). Sham-operated embryos were processed in parallel with each group of experimental embryos. Fifteen experimental and 16 control embryos were used. On being